# PINE BLUFF ARSENAL

## PINE BLUFF, ARKANSAS

## TECHNICAL REPORT QAL-90-1

## AN EVALUATION OF A LIQUID CHROMATOGRAPHIC METHOD FOR THE PURITY ASSESSMENT OF SOLVENT YELLOW 33



BY

RAY L. DAVIS
RONALD W. WISE

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Ray L. Davis; Ronald W. Wise

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An ~~HPLC~~ method for analysis of Solvent Yellow 33 purity is investigated, developed and tested. The HPLC method is found to be more accurate and more precise than earlier methods. In addition, the automated HPLC system is less time intensive and therefore more cost efficient. The method involves analysis in methanol solution on a reverse phase C-18 Column using a UV detector set at 429nm.

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AN EVALUATION OF

A LIQUID CHROMATOGRAPHIC METHOD

FOR THE PURITY ASSESSMENT OF SOLVENT YELLOW 33

APPROVED:

Patrick E. Brown

PATRICK E. BROWN, GS-12
Ch, Chemical Analysis Branch, PAD

1/9/90
DATE

Steven D. Lowrey

STEVEN D. LOWREY, GM-13
Ch, Fixed & Mobile Lab Br, PAD

1/9/90
DATE

H. Lawson Love

H. LAWSON LOVE, GM-13
Director, Product Assurance

1/9/90
DATE

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## 1. EXECUTIVE SUMMARY:

A new method for the assessment of solvent yellow 33 (SY-33) purity was evaluated for precision and accuracy (P&A). Four analysts performed the procedures on four different days. The P&A data indicate that the new method is accurate to three significant digits and has a precision of ± 0.76% for the purity analysis of solvent yellow 33 in any homogeneous container of the dye. Considering that the current procedure outlined in DOD-D-51485 has been difficult to perform and keep in statistical control, it is recommended that this new method be adopted for all purchases of this dye by the Pine Bluff Arsenal.

## 2. INTRODUCTION:

The current method for solvent yellow 33 (SY-33) purity analysis stipulated in DOD-D-51485 is in need of revision. This method is thought inadequate because the dilution scheme is labor intensive and time consuming. In addition, the analytical results often vary widely from one laboratory to another, and from one HPLC operator to another[1]. The precision of the current method is typically unacceptable, (the uncertainty is often ± 3% or more depending upon the analyst and the equipment used). As a result, a need exists to simplify the dilution scheme and reduce the method inherent variance.

A one step dilution procedure is recommended in which SY-33 is accurately weighed and quantitatively transferred into Class A volumetric glassware. The sample can be prepared in a shorter period of time with fewer errors because dilution steps are not required. The revised method should also exhibit less inherent variance. However, certain evaluations must be made before the revised method is ready for release for general use. Enough data must be gathered to allow a statistical analysis of the precision and accuracy of the method. A well designed P&A plan will provide results that indicate whether the method is adequate to meet the needs of the customer. In order to write purity specifications which assure the purchase of acceptable dyes, the precision and accuracy by which laboratories can be expected to test the SY-33 purity must be known.

The following report presents the results of an intralaboratory evaluation of a new liquid chromatographic method for the purity assessment of solvent yellow 33 (CI# 47000). The report also presents a comprehensive explanation of the laboratory techniques involved in the sample preparation for dye purity assessment.

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[1] Pine Bluff Arsenal Technical Report PBATR QAL90-2.

## 3. MATERIALS:

### 3.1 Instrumentation:

HPLC analysis was carried out using a Waters model 490 UV/Vis detector. Samples were injected using a LDC model 713 autosampler fitted with a 10 µL fixed loop Rheodyne air actuated injector. Peak areas were quantified using an LDC model CI-10 integrator. Weight measurements were made with a Perkin-Elmer AD-4 Autobalance for the data obtained during days 1 and 3. A Sartorias R-160-P research balance was used to obtain the data for days 2 and 4.

### 3.2 Chemicals:

Methanol and water were B&J Brand HPLC grade. The solvent yellow 33 (R9913-26) was from American Cyanamid Co., Wayne NJ. The purity of the dye was determined by a comparison with a primary standard prepared by the Chemistry Department, NCTR, Jefferson, AR. (Pine Bluff Arsenal Technical Report PBATR QAL90-2).

## 4. EXPERIMENTAL METHODS:

Purity assessment of highly pure material with high accuracy and precision is a uniquely difficult analytical problem. For that reason, techniques used in this study to reduce systematic errors which may be overlooked as insignificant in other situations have been presented in detail.

### 4.1 Precision and Accuracy Study Design:

The study was conducted over a period of four days and involved four different analysts. Representative samples of known SY-33 purity are analyzed on each of the four days. Each analyst was assigned a testing day to prepare a fresh SY-33 calibration curve, make the appropriate sample dilutions, mix fresh mobile phase solutions, and operate the HPLC instrumentation. The nominal sample mass was set at 10 mg of material which was then dissolved into 100 ml of HPLC grade methanol. A calibration curve was prepared from standard R9913-26 by weighing approximately 9, 10, and 11 mg prepared in singlet and injected in duplicate at the beginning of the analysis. All weight measurements are determined to the nearest 0.01 mg. Three test samples were prepared from R9913-26 and given to the analyst labeled S-1, S-2 and S-3. Each of the test samples were weighed out in triplicate and injected in duplicate. One standard is injected on the HPLC after every 6 sample injections. The

agreement between standards is determined from their respective response factors. The correlation coefficient for the calibration curve is calculated. The percent variance between the observed and theoretical concentrations is calculated. The confidence interval (i.e. the uncertainty in measurements) expected from use of this method for purity determination of SY-33 is assessed.

4.2 Laboratory Techniques:

Calibration standards are prepared to bracket the target concentration of the test solutions. The nominal concentrations for the standard solutions are 90, 100, and 110 µg/ml (i.e. the concentration units of micrograms per milliliter). All mass measurements should be read to within ± 0.2% of the nominal value. For example, a 5 place (the number of places past the decimal point) research grade balance is required to measure the mass range between 5 to 500 milligrams, while a 4 place analytical balance may be used to measure 50 to 500 milligrams. If high precision balances are not already in place, it is advised that the testing facility upgrade at the earliest available opportunity. Balances capable of accurately weighing milligram (mg) samples are commercially available for about $4000[2].

Ten grams of the test material is placed in a 100 ml beaker and thoroughly mixed to insure homogeneity. Motor driven stirrers capable of variable speeds between 500 to 7500 rpm have been found satisfactory for this purpose. Prefabricated weighing boats made from inert materials such as glass, polyethylene, polypropylene or PTFE may be used to weigh dye samples. Clean, dry tweezers must be used exclusively to handle the weighing boats. The empty boat is placed on the balance pan and the balance is tared to zero. The boat is removed from the pan to ensure that none of the dye accidentally spills onto the balance pan. The sample is placed in the weigh boat with a clean dry scoopula. The boat containing the sample is then returned to the pan and the mass measurement is made. Additional sample may be added to the weigh boat to bring the final measurement to the desired mass. Unused and/or potentially contaminated material should never be returned to the primary container.

The sample can be directly deposited in the volumetric flask. Small samples (e.g. 50 mg or less) may be weighed on a thin piece of aluminum foil (approximate dimensions 2cm X 2cm) and inserted directly into an appropriately sized volumetric flask. Care should be taken to insure that all of the aluminum weigh boats have a near equivalent size and weight. Methanol is added until

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[2] 1988 Fisher Scientific Catalog, page 54.

the volumetric flask is about 90% full. Alternatively, the quantitative transfer of the sample is easily accomplished using the apparatus shown in Figure 1. A glass powder funnel is placed directly above a Class A volumetric flask of appropriate size. The weigh boat containing the sample is placed in the powder funnel and thoroughly flushed with HPLC grade methanol. As with the preceding technique, flushing continues until the volumetric flask is about 90% full.

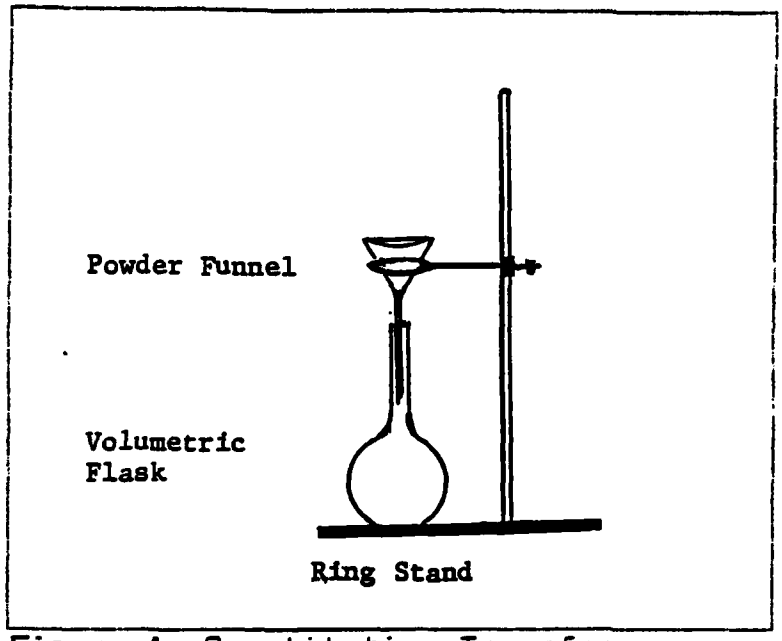


Figure 1 Quantitative Transfer of SY-33.

The volumetric flask is then sonicated in an ultrasonic bath for one minute. Most Class A volumetric glassware is calibrated at twenty degrees Centigrade. As a result, it may be necessary to submerge the volumetric flask in a water bath maintained at 20° C to obtain accurate volume measurements. Once thermal equilibrium is reached, the flask is filled to the calibration mark with methanol, capped, and inverted twenty times to insure proper mixing. Aliquotes may be transferred into HPLC autosampler vials using a Pasteur pipet. The vials must be immediately sealed to reduce the likelihood of solvent evaporation.

The HPLC mobile phase is composed of 90% methanol and 10% water. A typical preparation involves pouring 900 ml of HPLC grade methanol into a 1000 ml graduated cylinder. 100 ml of high purity water (e.g. MilliQ or HPLC grade water) is then added to the graduated cylinder. The resulting solution is drawn through a Nylon 66, 0.2 micron, filter as shown in Figure 2. The filtered liquid is poured into a 1000 ml stock bottle containing a clean stir bar. The solution is stirred and subjected to a vacuum of at least 15 inches mercury. The degassing process should be continued until bubbles can no longer be observed in the mobile phase solution (approximately 20 minutes). The filtered liquid intake line on the HPLC is then placed in the mobile phase solution and the pump is set for a flow rate of 1 ml/minute. The mobile phase container is sealed with parafilm to reduce the likelihood of contamination and to retard the evaporation of the volatile components.

The HPLC should be fitted with a 4.6 X 250 mm Brownlee OD-5A

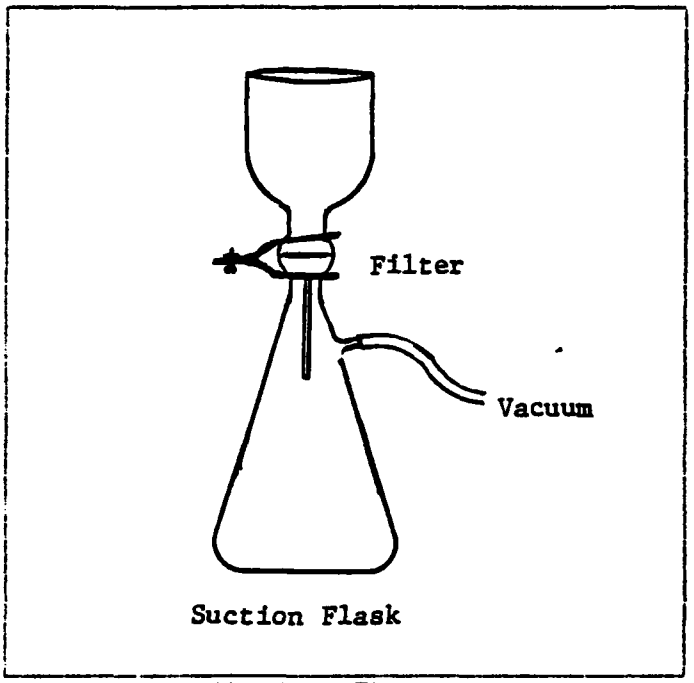


Figure 2 Mobile Phase Filtration.

Spheri-5 RP-18 column and a 15 X 3.2 mm 7 micron RP-18 New Guard guard column. An equivalent guard column-column arrangement may be substituted. A dependable autosampler using fixed loop injection is recommended. Manual injections may be made, but manual injections can be subject to a loss in reproducibility. A 10 µl loop is used with both manual and automatic HPLC injection modes. A suitable UV-Vis detector set at 429 nm is required. The mobile phase should be flushed through the HPLC for about 30 minutes at a flow rate of 1 ml/min to obtain instrumental stability.

Noise measurements should be made to insure that the HPLC is operating properly. Since each detector/integrator combination produces different response values, it is advised that an assessment of the unit's minimal noise value be obtained prior to any analysis. This is accomplished by flushing the mobile phase liquid through the HPLC and periodically checking the noise response value. Consult the integrator owner's manual for specific guidance in obtaining these values.

The analyte retention time should be about 7 minutes using the specified HPLC system. A minor contaminant found in the standard material will appear in the tailing section of the analyte peak with a retention time near 9.5 minutes. This peak must be skimmed off the trailing edge of the analyte peak to obtain accurate measurements. The skimmed peak area provides a useful reference which insures that the integrator responses are consistent from one laboratory to another. The operator may wish to begin collecting data about 5 minutes after the injection is made in order to conserve integrator memory and/or strip chart paper. The data collection process can normally be halted 11 minutes after the injection is made.

The instrument should be equilibrated by injecting the middle standard solution (i.e. 100 µg/ml) repeatedly. Peak area measurements should not vary more than ± 0.3%. When the last three injections of the middle standard solution meet this criterion, the instrument is properly equilibrated.

## 4.3 Data Handling and Equations:

Quality control for the preparation of standard solutions is maintained by evaluating the response factors for each of the calibration solutions. Duplicate injections are made for each of the three standard solutions in order to reduce the effects of instrumental variance; thus, allowing for a more accurate assessment of the variance derived from the solution preparation process. Response factors are calculated using Equation 1 below. The response factor value for each calibration standard solution should agree with all others within 1.0%. Equation 2 provides the mathematical relationship needed to evaluate response factor agreement between calibration standards. Standard solutions which do not meet these criterion should be reweighed until a suitable set of calibration solutions are obtained.

EQUATION 1: rf = Peak Area / concentration (µg/ml)

EQUATION 2: % Agree = $[(rf_h - rf_l) / rf_m] * 100$

where $rf_h$ is the highest average response factor value,
$rf_l$ is the lowest average rf value,
and $rf_m$ is the mean response factor value.

Some of the preliminary data obtained using the reverse phase HPLC method have been compiled in Table 1.

TABLE 1. Calibration Data for SY-33
(Each standard was injected twice)

| Standard Conc. (µg/ml) | HPLC Response (Peak Area) | Average Response factor |
|---|---|---|
| 86.33 | 216474 | |
| | 216392 | 2507.04 |
| 101.75 | 255637 | |
| | 255335 | 2510.92 |
| 111.03 | 278332 | |
| | 278563 | 2507.86 |

The data presented in Table 1 were obtained from the PBA PAD log book AAC08, page 114; reference sample numbers 113E, 113F and

113D respectively. The agreement between these standards is calculated as follows:

$$\% \text{ Agree} = [(2510.92 - 2507.04) / 2508.61] * 100 = 0.15\%$$

where the mean response factor value ($rf_{\bar{x}}$) is calculated as
(2507.04 + 2510.92 + 2507.86) / 3 = 2508.61

The purity of unknown samples is determined from the resulting calibration curve. Suitable curve fitting software is commercially available for most personal computers and several hand held calculators provide linear regression and correlation functions. A first approximation of the best fit line is to assume that the y intercept is zero. Calculations can then be made using the straight line equation $y = mx + b$, where b is the y intercept and m is the slope of the curve. When the intercept, b, is zero, the slope, m, is given as $y / x$. By choosing y as the peak area response and x as the concentration term, m is simply the mean response factor value ($rf_{\bar{x}}$). The response factor for an unknown sample is calculated from peak area and solution concentration as shown in Equation 3. The percent purity of SY-33 is related to the response factors as stated in Equation 4.

EQUATION 3: $rf_u = PA_u / C_u$

where $PA_u$ is the peak area for the unknown and
$C_u$ is the solution concentration for the unknown. This value is simply the mass of the unknown sample (in µg) divided by the dilution volume (in ml).

EQUATION 4: $\% P_u = (rf_u / rf_s) * \% P_s$

where $\% P_u$ is the purity of the unknown SY-33 sample,
$\% P_s$ is the purity of the standard SY-33,
$rf_s$ is the mean response value of standards as defined above,
and $rf_u$ is defined by Equation 3 above.

The response values for analytical instrumentation tend to drift or change with time. HPLC is no exception. It is necessary to reinject a calibration standard periodically in order to evaluate

this phenomenon. The middle (100 µg/ml) calibration standard is analyzed after every six sample injections. This standard is treated as an unknown and must fall within ± 0.5% of the known purity before the sample results can be considered valid. Otherwise, all of the calibration standards must be reinjected to establish a new $rf_x$ value. In addition, the last six unknown samples must be reanalyzed to assure their accurate purity determination. Typical instrumental drift has been measured at 0.3% over a period of 8 hours using this method (e.g. PBA PAD log book AAC08; page 64). Drift of such small magnitude will not often require instrumentation recalibration, but is sufficient to warrant periodic observation.

The instrumental results obtained in this study are reported in terms of observed concentrations, which are determined from the applicable calibration curve. The difference between the observed concentration value and the theoretical concentration provides a convenient yard stick to measure the variance of the method as shown in Equation 5.

EQUATION 5: $V\% = [(TC - OC) / TC] * 100$

where V% is the percentage variance from the theoretical concentration,
TC is the theoretical concentration,
and OC is the observed concentration.

The deviation from the predicted value is partly composed of the instrumental errors and solution preparation errors. The former type of error results from a variety of sources including HPLC drift, random fluctuations in the detector response, and errors in the calibration curve determinations. The latter form of error may be related to sample homogeneity, mass measurements, sample transfer efficiency, volume measurements and the sample dilution efficiency. For example, failure to completely mix the test sample may increase the variance for purity determinations. Any spillage of the sample during the transfer to the volumetric flask will adversely affect the accuracy and reproducibility of the mass measurements. Erroneous volume measurements may occur from reading the meniscus at a poor angle of observation or from making the measurements at different solution temperatures. Failure to sonicate the sample will leave undissolved material on the bottom of the volumetric flask. The solution preparation error is also influenced by the capabilities of the analytical balance used to obtain the mass measurements. As a result, most of the solution preparation errors may be avoided or controlled by the analyst and for the most part, represent human errors.

The magnitude of the instrumental error may be evaluated since duplicate injections were made for each sample aliquot. The

standard deviation estimate, $s_d$, for paired observations may be determined from Equation 6. A more accurate estimate of the standard deviation value for HPLC analysis may be obtained by pooling the individual $s_d$ values using Equation 7. The pooled standard deviation value can be used to determine the uncertainty (U) in sample measurements at any desired confidence level for the proposed HPLC method as shown by Equation 8.

EQUATION 6: $s_d = [\Sigma d^2 / (2 * q)]^{0.5}$

where $s_d$ is the estimated standard deviation,
$d$ is the difference in the duplicated measurements,
and $q$ is the number of sets of duplicate measurements.

EQUATION 7:

$$s_p = [(v_1 s_{d1}^2 + v_2 s_{d2}^2 + \ldots + v_t s_{dt}^2) / (v_1 + v_2 + \ldots + v_t)]^{0.5}$$

where $s_p$ is the pooled standard deviation value based on $(v_1 + v_2 + \ldots + v_t)$ degrees of freedom.
$s^2$ is the estimated standard deviation value for a given set of measurements,
$v$ is the degrees of freedom for a set of measurements [v=(n-1) where n is the number of measurements made].

An estimation of the sample preparation error is obtained by subtracting the uncertainty in measurements attributed to the HPLC instrumental error from the total uncertainty in measurements. The total uncertainty value (U) is calculated from Equation 8 using the standard deviation ($\sigma_{n-1}$) of the average V% values given in Table 3.

EQUATION 8: $U = \pm (t * s_d) / (n^{0.5})$

where $U$ is the uncertainty in the sample measurement,
$n$ is the number of samples,
$s_d$ is the estimated standard deviation,
and $t$ is the student t value determined at the 95% confidence level. For 3 data points, t = 4.303. The value of t becomes smaller as the number of data points increase.

## 5. RESULTS AND DISCUSSION:

The calibration curves obtained in this four day precision and accuracy study are shown in Figures 3, 4, 5 and 6. The correlation coefficients are 0.9998, 0.9997, 0.9995 and 0.9998 respectively. The mean response factors and the corresponding standard deviation (n-1) are 2498 ± 0.2%, 2498 ± 0.4%, 2511 ± 0.4% and 2509 ± 0.7%, respectively. Analyst #4 inadvertently injected the standards once each. Response factors and their % agreement are shown in Table 2. Except for day #4, all calibration samples agreed within 1.0%.

The data acquired by the duplicate injections of three test samples prepared in triplicate (total of nine preparations and 18 injections per day) is shown in Table 3. The percent variance (V%) for each of the individual samples was obtained by taking the average of the percent variances of the duplicate injections. The negative signs indicate that the determined purity is less than the theoretical value. The signed values are used in calculating the averages. The daily grand average percent variance and standard deviation ($\sigma_{n-1}$) values shown in Table 3 were calculated using the nine average percent variances (with the exception of day #1 which only used the last eight average percent values). The average percent variance over the entire four day period was +0.078%. This value was determined by obtaining the mean of the 4 daily grand averages [i.e. $V\%_{\bar{x}}$ = (0.076 + 0.272 - 0.165 + 0.130) / 4]. The positive sign indicates that the experimentally obtained purity values are greater than the theoretical purity predictions by 0.078%. The overall accuracy for the method is directly related to the ideal results (100%) plus the signed percent deviation (i.e. 1 + 0.00078 = 1.00078). The overall accuracy for the method is exceptionally good since the estimated accuracy value (i.e. 1.00078) closely approximates unity. These results imply that there are no systematic errors inherent in this method.

The uncertainty in a given sample measurement (U) can be divided into two measurable components. The uncertainty associated with the instrumentation ($U_{inst}$) and the uncertainty associated with the sample preparation process ($U_{sp}$). The former measurement, $U_{inst}$, represents the smallest amount of uncertainty that the method can obtain using the specified instrumentation. The HPLC instrumental uncertainty for a given sample is estimated at ± 0.4994% and was derived by pooling the standard deviation values provided in Table 4. The instrumental uncertainty may have been overestimated since nonhomogeneity in the test solutions can also contribute to the variance. Evidence to this effect may be obtained from the data acquired on day 1 of the P&A study. In this case, the uncertainty derived from all sources is less than the estimated instrumental uncertainty value (i.e. the standard deviation value for day 1 is 0.1321 which produces an estimated uncertainty of ± 0.3282%). The analyst from day 1 suggests that

the volumetric flask be inverted several times to insure proper mixing just before the HPLC samples are removed. Precision is improved by filling the Pasteur pipet with sufficient volume to fill both HPLC autosampler vials. The data from day 1 suggests that the accuracy for the method is not adversely affected by employing these techniques.

The instrumental uncertainty, $U_{HPLC}$, as estimated from the pooled standard deviation values provided in Table 4 is calculated as follows:

$$U_{HPLC} = \pm [(4.303 * 0.0201) / (3^{0.5})] = \pm 0.04994$$

The uncertainty should be expressed as a percent of the nominal mass (10 mg) for comparison purposes.

$$U_{\%HPLC} = \pm (0.04994/10) * 100 = \pm 0.4994\%$$

The uncertainty associated with the sample preparation process ($U_{SP}$) is estimated by subtracting the instrumental uncertainty ($U_{\%HPLC}$) from the total method uncertainty (U). The total uncertainty for in a given sample measurement (U) is obtained from the standard deviation ($\sigma_{n-1}$) values provided in Table 3. The resulting standard deviation is expressed as a percentage and may be directly compared to $U_{\%HPLC}$. As before, these values are pooled to provide a more accurate estimate of the standard deviation. The total uncertainty for the method is estimated as ± 0.7587%.

$$U = \pm [(4.303 * 0.3054) / (3^{0.5})] = \pm 0.7587\%$$

The uncertainty associated with the sample preparation process ($U_{SP}$) is estimated as the difference between U and $U_{\%HPLC}$.

$$U_{SP} = U - U_{\%HPLC} = \pm 0.2593\%$$

A typical analysis scheme calls for 3 aliquotes of dye to be removed from a larger container, such as a 55 gallon drum, and analyzed for purity. As a result, the true purity of the dye in the container should routinely be estimated within ± 0.76% of the analytically determined value. This represents a significant improvement over the previous method which exhibited on overall uncertainty that tended to range between ± 1.5% and ± 3%. The results from each day of the P&A study are summarized in Table 5.

## 6. RECOMMENDATIONS:

As a result of the evaluation of this new liquid chromatographic method for purity assessment of solvent yellow 33, we recommend it to be adopted for all future purchases of this dye by the Pine Bluff Arsenal. The proposed method is capable of determining the purity of the SY-33 samples with greater accuracy and precision than any other method known to the writers. The proposed method is considerably more efficient and requires about one half as much time to obtain valid purity results as the current procedure. We also suggest that this method be evaluated for interlaboratory "ruggedness" before criteria for pass or fail of lot shipments is finalized.

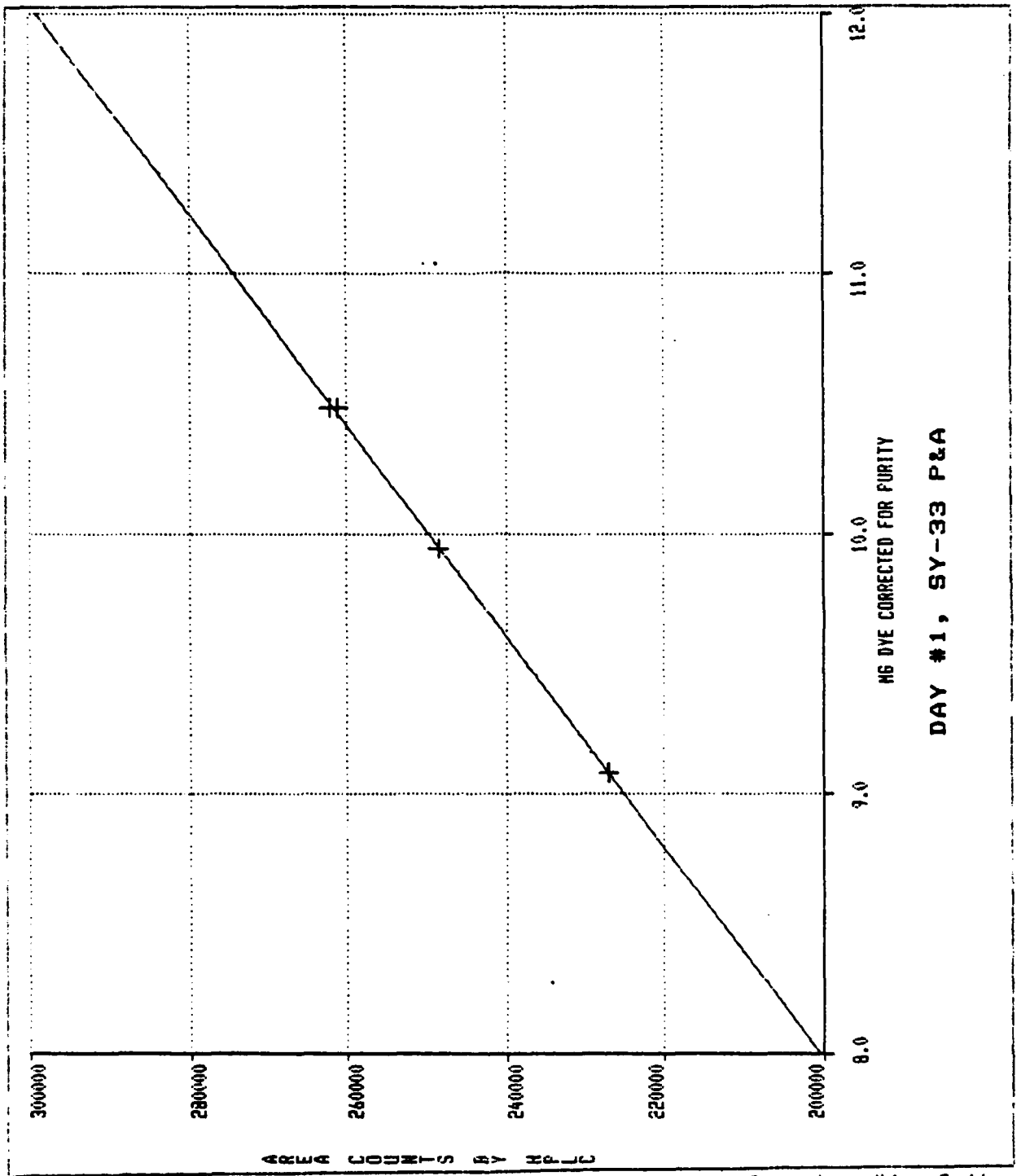


**Figure 3** Solvent Yellow 33 calibration curve for day #1 of the P&A study. The correlation coefficient for this curve is 0.9998. The curve has a Y intercept of 3632 and a slope of 24609.

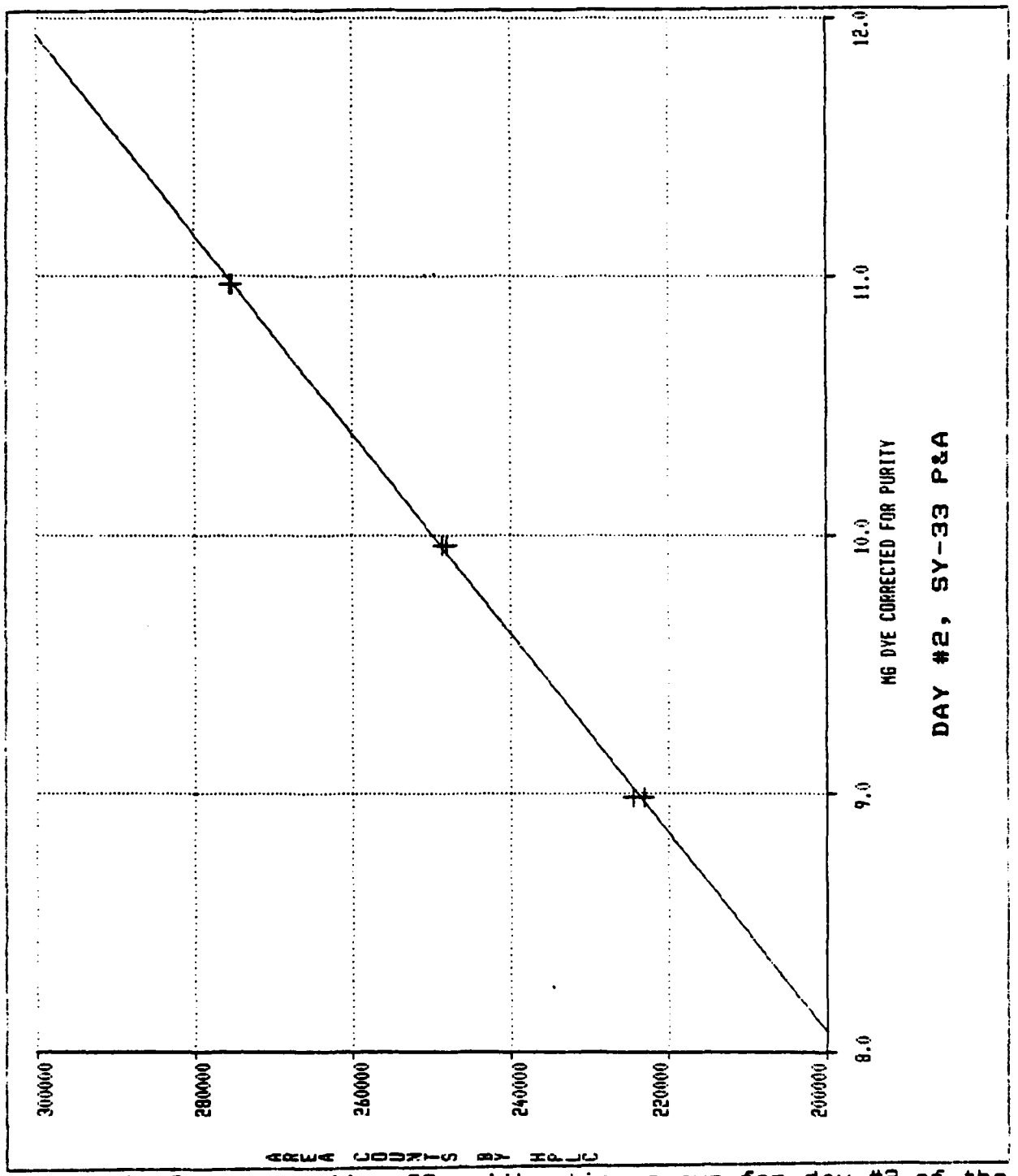


**Figure 4** Solvent Yellow 33 calibration curve for day #2 of the P&A study. The correlation coefficient for this curve is 0.9997. The Y intercept is -9477 and the slope is 25933.

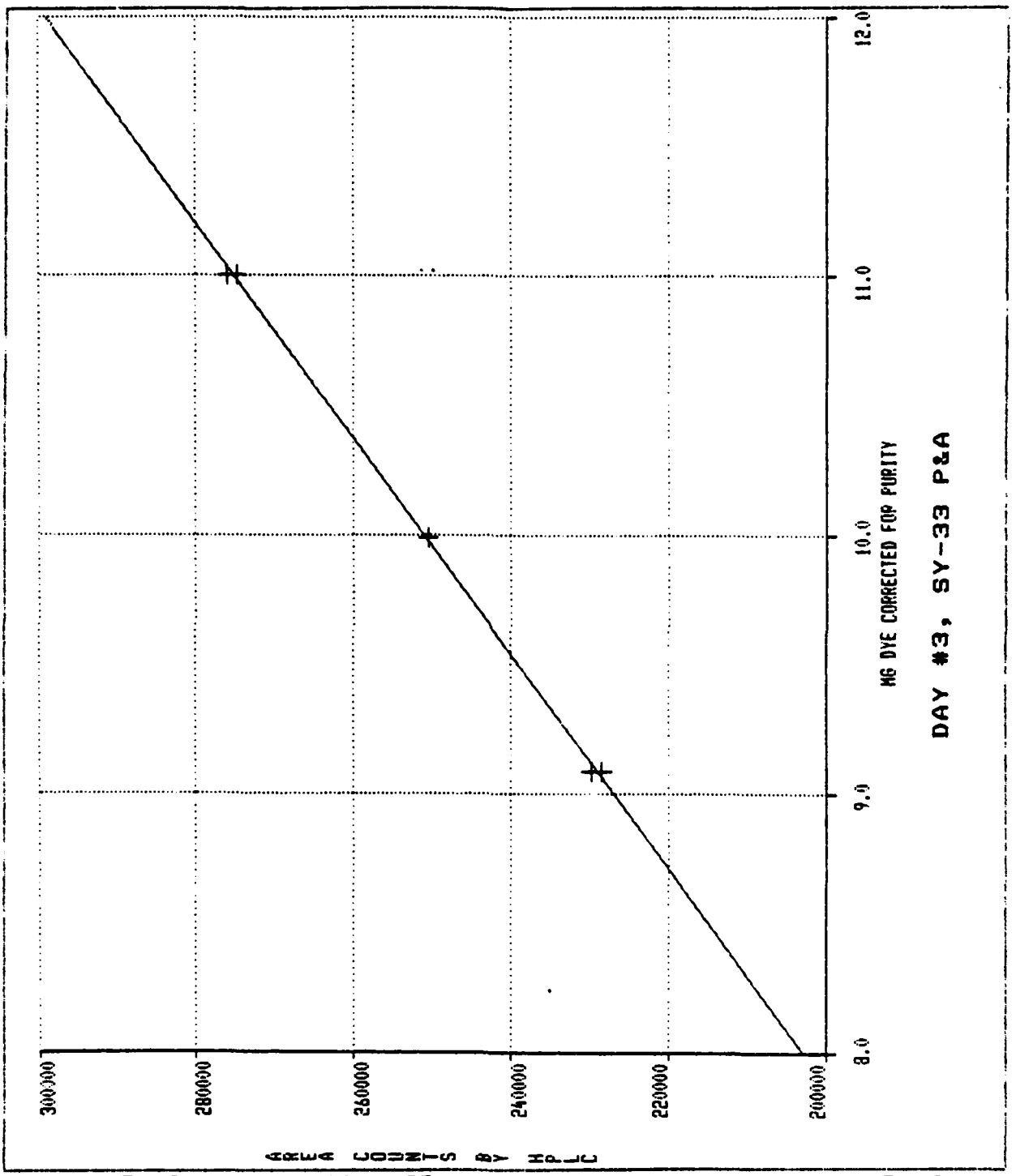


**Figure 5** Solvent Yellow 33 calibration curve for day #3 of the P&A study. The correlation coefficient for this curve is 0.9995. The Y intercept is 10405 and the slope is 24061.

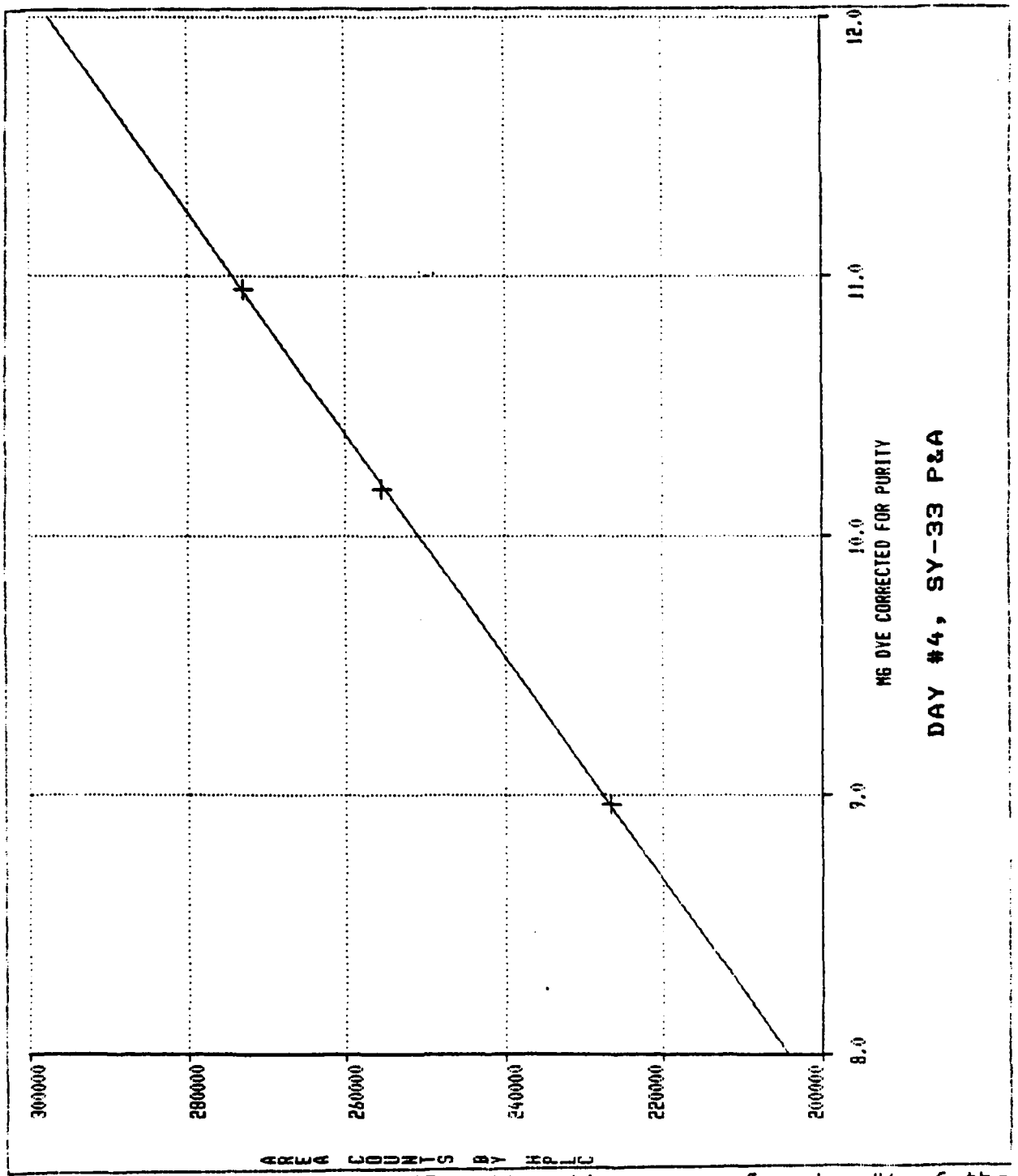


**Figure 6** Solvent Yellow 33 calibration curve for day #4 of the P&A study. The correlation coefficient for this curve is 0.9998. The Y intercept is 17973 and the slope is 23289.

TABLE 2. P&A CALIBRATION DATA

| Calibration Curve | Dye Conc. (µg/mL) | Peak Area | Response Factor | Average R.F. |
|---|---|---|---|---|
| Day 1: | 90.82 | 227175 | 2501.4 | |
| | | 227024 | 2499.7 | 2500.5 |
| | 99.43 | 248415 | 2498.4 | |
| | | 248417 | 2498.4 | 2498.4 |
| | 104.87 | 261198 | 2490.7 | |
| | | 262112 | 2499.4 | 2495.0 |
| | | | % Agree = 0.22% | |
| Day 2: | 89.86 | 224490 | 2498.2 | |
| | | 223073 | 2482.5 | 2490.3 |
| | 99.60 | 248078 | 2490.7 | |
| | | 248649 | 2496.5 | 2493.6 |
| | 109.74 | 275176 | 2507.5 | |
| | | 275477 | 2510.3 | 2508.9 |
| | | | % Agree = 0.74% | |
| Day 3: | 90.86 | 229871 | 2529.9 | |
| | | 228586 | 2515.8 | 2522.9 |
| | 99.90 | 250351 | 2506.0 | |
| | | 250411 | 2506.6 | 2506.3 |
| | 110.02 | 274655 | 2496.4 | |
| | | 275959 | 2508.3 | 2502.3 |
| | | | % Agree = 0.82% | |
| Day 4: | 89.66 | 226590 | 2527.2 | |
| | 101.79 | 255534 | 2510.4 | |
| | 109.54 | 272779 | 2490.2 | |
| | | | % Agree = 1.47% | |

TABLE 3. P&A DATA

| Theoretical Conc. (µg/ml) | Observed Conc. (µg/ml) | Percent Error in Measurements (V%) | Average V% |
|---|---|---|---|
| 10.967 | 10.7980 | -1.5410 | |
| 10.967 | 10.8012 | -1.5118 | -1.5264[a] |
| 9.997 | 10.0044 | 0.0740 | |
| 9.997 | 10.0203 | 0.2331 | 0.15355 |
| 10.051 | 10.0609 | 0.0985 | |
| 10.051 | 10.0745 | 0.2338 | 0.16615 |
| 9.943 | 9.9698 | 0.2694[b] | |
| 9.998 | 9.9935 | -0.0450 | |
| 9.998 | 10.0007 | 0.0270 | -0.00900 |
| 10.086 | 10.0720 | -0.1388 | |
| 10.086 | 10.0826 | -0.0337 | -0.08625 |
| 9.947 | 9.9462 | -0.0080 | |
| 9.947 | 9.9586 | 0.1166 | 0.01720 |
| 9.943 | 9.9656 | 0.2269 | |
| 10.101 | 10.0927 | -0.0822 | |
| 10.101 | 10.0938 | -0.0713 | -0.07675 |
| 9.948 | 9.9522 | 0.0422 | |
| 9.948 | 9.9783 | 0.3046 | 0.17340 |
| 9.946 | 9.9618 | 0.1589 | |
| 9.946 | 9.9842 | 0.3841 | 0.27150 |
| 9.943 | 9.9688 | 0.2596 | |
| Day 1 Mean: | | | 0.07623 |
| Standard Deviation ($\sigma_{..1}$): | | | 0.13212 |

[a] An error was observed in the preparation of this sample prior to analysis. The sample was weighed on a 2 cm X 2 cm square of aluminum foil and placed in a powder funnel as shown in Figure 1. The sample was then thoroughly washed with HPLC grade methanol as outlined above. The aluminum foil was allowed to dry and examined under 1.25 X magnification. SY-33 material was observed as an evenly dispersed film which coated the aluminum foil surface. From this observation, it was concluded that the flushing procedure was inadequate for samples weighed on aluminum foil and that all other such samples should be placed directly into the appropriate volumetric flask. The data obtained from this measurement is excluded from the calculation of the total method uncertainty (U) based on the Dixon test for outlying observations. Rejection is made at the 99% confidence level.

[b] The middle standard is reinjected to assess HPLC drift.

TABLE 3. P&A DATA (Continued)

| Theoretical Conc. (µg/ml) | Observed Conc. (µg/ml) | Percent Error in Measurements (V%) | Average V% |
|---|---|---|---|
| Day 2: | | | |
| 9.970 | 9.9400 | -0.3009 | |
| 9.970 | 9.9410 | -0.2909 | -0.29590 |
| 9.960 | 10.0200 | 0.6024 | |
| 9.960 | 10.0180 | 0.5823 | 0.59230 |
| 9.940 | 9.9780 | 0.3823 | |
| 9.940 | 9.9770 | 0.3722 | 0.37725 |
| 9.960 | 9.9424 | -0.1767 | |
| 9.950 | 9.9100 | -0.4020 | |
| 9.950 | 9.9070 | -0.4322 | -0.41710 |
| 9.940 | 9.9610 | 0.2113 | |
| 9.940 | 9.9800 | 0.4024 | 0.30685 |
| 9.970 | 10.0440 | 0.7422 | |
| 9.970 | 10.0400 | 0.7021 | 0.72215 |
| 9.960 | 9.9326 | -0.2751 | |
| 9.950 | 9.9530 | 0.0302 | |
| 9.950 | 9.9750 | 0.2513 | 0.14075 |
| 9.970 | 10.0390 | 0.6921 | |
| 9.970 | 9.9730 | 0.0301 | 0.36110 |
| 9.950 | 10.0080 | 0.5829 | |
| 9.950 | 10.0230 | 0.7337 | 0.65830 |
| 9.960 | 9.9502 | -0.0984 | |
| Day 2 Mean: | | | 0.27174 |
| Standard Deviation ($\sigma_{n-1}$): | | | 0.40145 |

TABLE 3. P&A DATA (Continued)

| Theoretical Conc. (µg/ml) | Observed Conc. (µg/ml) | Percent Error in Measurements (V%) | Average V% |
|---|---|---|---|
| Day 3: | | | |
| 10.103 | 10.0730 | -0.2969 | |
| 10.103 | 10.0680 | -0.3464 | -0.32165 |
| 9.909 | 9.9140 | 0.0505 | |
| 9.909 | 9.8700 | -0.3936 | -0.17155 |
| 9.809 | 9.7750 | -0.3466 | |
| 9.809 | 9.8320 | 0.2345 | -0.05605 |
| 9.990 | 9.9903 | 0.0034 | |
| 10.165 | 10.1530 | -0.1181 | |
| 10.165 | 10.1210 | -0.4329 | -0.27550 |
| 10.025 | 10.0040 | -0.2095 | |
| 10.025 | 9.9660 | -0.5885 | -0.39900 |
| 10.161 | 10.0830 | -0.7676 | |
| 10.161 | 10.1270 | -0.3346 | -0.55110 |
| 9.990 | 9.9859 | -0.0410 | |
| 10.118 | 10.1530 | 0.3459 | |
| 10.118 | 10.2020 | 0.8302 | 0.58805 |
| 10.068 | 10.0790 | 0.1093 | |
| 10.068 | 10.0600 | -0.0795 | 0.02980 |
| 10.090 | 10.0430 | -0.4658 | |
| 10.090 | 10.0700 | -0.1982 | -0.33200 |
| 9.990 | 9.9992 | 0.0921 | |
| Day 3 Mean: | | | -0.16544 |
| Standard Deviation ($\sigma_{n-1}$): | | | 0.33268 |

TABLE 3. P&A DATA (Continued)

| Theoretical Conc. (µg/ml) | Observed Conc. (µg/ml) | Percent Error in Measurements (V%) | Average V% |
|---|---|---|---|
| Day 4: | | | |
| 10.189 | 10.1790 | -0.0981 | |
| 10.189 | 10.1590 | -0.2944 | -0.19625 |
| 10.248 | 10.2180 | -0.2927 | |
| 10.248 | 10.2480 | -0.0000 | -0.14635 |
| 10.258 | 10.2670 | 0.0877 | |
| 10.258 | 10.2390 | -0.1852 | -0.04875 |
| 10.179 | 10.2094 | 0.2987 | |
| 10.248 | 10.3060 | 0.5660 | |
| 10.248 | 10.2540 | 0.0585 | 0.31225 |
| 10.189 | 10.2330 | 0.4318 | |
| 10.189 | 10.2380 | 0.4809 | 0.45635 |
| 10.089 | 10.1260 | 0.3667 | |
| 10.089 | 10.1250 | 0.3568 | 0.36175 |
| 10.179 | 10.2042 | 0.2476 | |
| 10.039 | 10.0870 | 0.4781 | |
| 10.039 | 10.0730 | 0.3387 | 0.40840 |
| 10.069 | 10.0780 | 0.0894 | |
| 10.069 | 10.0220 | -0.4668 | -0.18870 |
| 10.119 | 10.1510 | 0.3162 | |
| 10.119 | 10.1300 | 0.1087 | 0.21245 |
| 10.179 | 10.1938 | 0.0015 | |
| Day 4 Mean: | | | 0.13013 |
| Standard Deviation ($\sigma_{n-1}$): | | | 0.27250 |

TABLE 4. HPLC VARIANCE DETERMINATION

| Observation 1 | Observation 2 | d | $d^2$ | $s_d$ |
|---|---|---|---|---|
| 10.7980 | 10.8012 | 0.0032 | 0.000010 | |
| 10.0044 | 10.0203 | 0.0159 | 0.000253 | |
| 10.0609 | 10.0745 | 0.0136 | 0.000185 | 0.00864 |
| 9.9935 | 10.0007 | 0.0072 | 0.000052 | |
| 10.0720 | 10.0826 | 0.0106 | 0.000112 | |
| 9.9462 | 9.9586 | 0.0124 | 0.000154 | 0.00728 |
| 10.0927 | 10.0938 | 0.0011 | 0.000001 | |
| 9.9522 | 9.9783 | 0.0261 | 0.000681 | |
| 9.9618 | 9.9842 | 0.0224 | 0.000502 | 0.01405 |
| 9.9400 | 9.9410 | 0.0010 | 0.000001 | |
| 10.0200 | 10.0180 | 0.0020 | 0.000004 | |
| 9.9780 | 9.9770 | 0.0010 | 0.000001 | 0.00100 |
| 9.9100 | 9.9070 | 0.0030 | 0.000009 | |
| 9.9610 | 9.9800 | 0.0190 | 0.000361 | |
| 10.0440 | 10.0400 | 0.0040 | 0.000016 | 0.00802 |
| 9.9530 | 9.9750 | 0.0220 | 0.000484 | |
| 10.0390 | 9.9730 | 0.0660 | 0.004356 | |
| 10.0080 | 10.0230 | 0.0150 | 0.000225 | 0.02905 |
| 10.0730 | 10.0680 | 0.0050 | 0.000025 | |
| 9.9140 | 9.8700 | 0.0440 | 0.001936 | |
| 9.7750 | 9.8320 | 0.0570 | 0.003249 | 0.02947 |
| 10.1530 | 10.1210 | 0.0320 | 0.001024 | |
| 10.0040 | 9.9660 | 0.0380 | 0.001444 | |
| 10.0830 | 10.1270 | 0.0440 | 0.001936 | 0.02709 |
| 10.1530 | 10.2020 | 0.0490 | 0.002401 | |
| 10.0790 | 10.0600 | 0.0190 | 0.000361 | |
| 10.0430 | 10.0700 | 0.0270 | 0.000729 | 0.02412 |
| 10.1790 | 10.1590 | 0.0200 | 0.000400 | |
| 10.2180 | 10.2480 | 0.0300 | 0.000900 | |
| 10.2670 | 10.2390 | 0.0280 | 0.000784 | 0.01864 |
| 10.3080 | 10.2540 | 0.0520 | 0.002704 | |
| 10.2330 | 10.2380 | 0.0050 | 0.000025 | |
| 10.1250 | 10.1250 | 0.0010 | 0.000001 | 0.02133 |
| 10.0870 | 10.0730 | 0.0140 | 0.000196 | |
| 10.0780 | 10.0220 | 0.0560 | 0.003136 | |
| 10.1510 | 10.1300 | 0.0210 | 0.000441 | 0.02508 |

TABLE 5. SUMMARY OF RESULTS

| Analyst | % Agree | $S_p$ HPLC | $U_{\%HPLC}$ | $U_{wp}$ | $\sigma_{n-1}$ | U |
|---|---|---|---|---|---|---|
| Day 1 | 0.22 | 0.01041 | 0.2586% | 0.070% | 0.1321 | 0.3282% |
| Day 2 | 0.74 | 0.01741 | 0.4325% | 0.596% | 0.4014 | 0.9973% |
| Day 3 | 0.82 | 0.02698 | 0.6703% | 0.156% | 0.3327 | 0.8265% |
| Day 4 | 1.47 | 0.02184 | 0.5426% | 0.134% | 0.2725 | 0.6770% |
| Pooled Totals: | | 0.02010 | 0.4994% | 0.259% | 0.3054 | 0.7587% |